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(54) Title: DNA BASE MISMATCH DETECTION USING FLOW CYTOMETRY

(57) Abstract

Flow cytometric DNA base mismatch detection. The use of immobilized mismatch-binding protein-coated microspheres to bind fluorescently labeled, mismatch-containing DNA for detection by flow cytometry is described. The genomic DNA of interest is made fluorescent by one of a number of techniques. The resulting fluorescent DNA is melted and allowed to reanneal. If the genomic sample contains a polymorphic site, the reannealed DNA will contain base mismatches. Microspheres bearing immobilized mismatch-binding protein are added to the DNA samples and the mismatch-containing DNA allowed to bind to the mismatch-binding proteins. The sample is then analyzed by flow cytometry to detect the fluorescent DNA bound to the microspheres. The present flow-cytometric method for mutation scanning has several important features: it is a single step, no wash assay, capable of rapid and sensitive analysis. It will allow amplified regions to be rapidly scanned for the presence of polymorphisms to prioritize samples for complete sequencing or to detect mutations in known regions of the genome.

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DNA BASE MISMATCH DETECTION USING FLOW CYTOMETRY

This patent application claims priority from Provisional Patent Application Serial No. 60/063,685 filed on October 28, 1997.

FIELD OF THE INVENTION

The present invention relates generally to the use of flow cytometry and immobilized mismatch binding proteins for the detection of DNA polymorphisms, including nucleotide polymorphisms, insertions, and deletions. This invention was made with government support under Contract No. W-7405-ENG-36 awarded by the U.S. Department of Energy to The Regents of The University of California. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

As the sequencing of the first human genome approaches completion, significant attention is being given to the evaluation of genetic variation among individuals. An important element of such an effort involves the identification of sites that vary from individual to individual, especially single nucleotide polymorphisms (SNPs). This information could be obtained from direct DNA sequencing of many individuals, but the low throughput and high cost of presently available sequencing procedures make this impractical on a large scale. As a result, considerable effort has been directed towards methods that "scan" DNA sequences for mutations before subjecting them to direct sequencing. These scanning methods have been the subject of frequent reviews. See, e.g., "Slowly But Surely Towards Better Scanning for Mutations," by Richard G.H. Cotton, Trends In Genetics **13**, 43 (1997). For high-throughput SNP discovery, a scanning method should be capable of rapid and parallel analysis with a minimum of sample preparation and handling. Gel-based methods offer some capacity for parallel analyses, but are very slow. High-performance liquid chromatography (HPLC) is often faster, but samples must be analyzed sequentially. Microplate-based assays require time-consuming sample processing, with many incubation and wash steps. However, these assays can

be performed in parallel, as can sample analysis. However, only one or two measurements can be made per microplate well.

A number of these methods are based on detecting differences in physical properties of polymorphism-containing DNA. The single-strand conformation polymorphism method (SSCP) relies on detecting sequence-related differences in the secondary structure of single-stranded DNA as detected by nondenaturing gel electrophoresis. Denaturing gradient-gel electrophoresis (DGGE) detects polymorphisms by the different mobilities of homoduplex versus mismatch-containing heteroduplex double-stranded DNA. The sensitivity of heteroduplex DNA to cleavage by chemicals (hydroxylamine/osmium tetroxide) or enzymes (T4 endonuclease VII or the *E.coli* Must/H/L complex) can be detected by gel electrophoresis.

Although gel electrophoresis is widely available, its use places limitations on analysis speed and throughput and there is significant interest in gel-free methods for mutation scanning. One example that has gained some popularity is the use of immobilized mismatch binding protein (IMP), typically the bacterial mismatch recognition protein MutS, to detect heteroduplex-containing DNA. This approach uses filter binding or microwell plate formats to screen for the presence of mismatch-containing heteroduplex in PCR amplified samples. In "Mutation Detection Using Immobilized Mismatch Binding Protein MutS," by Robert Wagner et al., Nucleic Acids Res. **23**, 3944 (1995), MutS immobilized by binding to nitrocellulose was found to provide a sensitive and accurate mutation detection assay. However, numerous washing steps are required during various steps in the assay. See, also "Allele Identification Using Immobilized Mismatch Binding Protein: Detection And Identification Of Antibiotic Resistant Bacteria And Determination Of Sheep Susceptibility To Scrapie," by P. Debbie et al., Nucleic Acids Res. **25**, 4825 (1997). Recently, the use of an immobilized mismatch cleaving protein to bind heteroduplex DNA for mutation detection has been reported. Endonuclease VII, which cleaves mismatch containing DNA in the presence of Mg^{++}, is immobilized on microtiter plates and used to bind

heteroduplex DNA in the absence of Mg^{++}. See, e.g., "Enzymatic Mutation Detection: Procedure For Screening And Mapping Of Mutations By Immobilization Endonuclease VII," by S. Golz et al., Nucleic Acids Res. **26**, 1132 (1998). Endonuclease VII has the advantage of recognizing all possible mismatches, including C/C mismatches, as well as bulges or loops caused by insertions or deletions. See, also "Detection of 81 Of 81 Known Mouse β-Globin Promoter Mutations With T4 Endonuclease VII-The EMC Method," by R. Youil et al., Genomics **32**, 431 (1996).

Flow cytometry provides multiparameter detection with excellent sensitivity in a homogenous assay format. Multicolor fluorescence detection can be exploited for the simultaneous detection of dozens, or potentially hundreds, of analytes in a single sample. These features enable washless, small volume, multiplexed analyses for high-throughput applications.

Accordingly, it is an object of the present invention to provide a method for detecting SNPs by using flow cytometry to locate mismatch-containing DNA strands attached to mismatch-binding proteins immobilized on microspheres.

Another object of the invention is to provide a high-throughput, washless, small volume method for detecting SNPs using flow cytometry.

Additional objects, advantages and novel features of the invention will be set forth in part in the description which follows, and in part will become apparent to those skilled in the art upon examination of the following or may be learned by practice of the invention. The objects and advantages of the invention may be realized and attained by means of the instrumentalities and combinations particularly pointed out in the appended claims.

SUMMARY OF THE INVENTION

To achieve the foregoing and other objects, and in accordance with the purposes of the present invention, as embodied and broadly described herein, the method for the detection of DNA polymorphisms hereof may comprise in combination: immobilizing a mismatch-binding protein on microspheres; preparing fluorescently labeled copies of the DNA to be interrogated; and flowing

the microspheres through a flow cytometer, wherein the binding of the fluorescent DNA to the microspheres is measured.

Preferably, the mismatch binding protein is the bacterial mismatch binding protein MutS or a homologue thereof.

motivation

Benefits and advantages of the present invention include a high-throughput, small volume, washless method for detecting SNPs in DNA.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and form a part of the specification, illustrate the embodiments of the present invention and, together with the description, serve to explain the principles of the invention. In the drawings:

FIGURE 1a is a schematic representation of molecules of MutS, which bind to SNP locations in DNA, immobilized on a microsphere in the presence of DNA strands having SNPs and strands of normal DNA, while FIG. 1b is a schematic representation of binding to the microsphere of the DNA strands which have SNPs as a result of the mismatch-binding protein immobilized thereon, normal DNA strands remaining in solution, whereby DNA having SNPs can be detected using flow cytometry.

FIGURE 2 is a graph of the fluorescence, as measured by flow cytometry, of immobilized mismatch-binding protein-coated microspheres as a function of the concentration of mismatch-containing DNA (open circles) or non-mismatch-containing DNA (black circles) after 30 min. of co-incubation.

DETAILED DESCRIPTION OF THE INVENTION

Briefly, the present invention includes the use of immobilized mismatch-binding protein-coated microspheres to bind fluorescently labeled, mismatch-containing DNA for detection by flow cytometry. The genomic DNA of interest is amplified by polymerase chain reaction (PCR) using fluorescently labeled nucleotide triphosphates. The resulting fluorescent DNA is melted and allowed to reanneal. If the genomic sample contains a polymorphic site, the reannealed DNA will contain base mismatches. Microspheres bearing immobilized

mismatch-binding protein are added to the DNA samples and the mismatch-containing DNA allowed to bind to the mismatch-binding proteins. The sample is then analyzed by flow cytometry to detect the fluorescent DNA bound to the microspheres. Alternatively, DNA may be incubated with soluble mismatch-binding protein, followed by immobilization of the DNA-mismatch-binding protein complex onto microspheres for analysis using flow cytometry. By adapting the assay to flow cytometry techniques, existing SNP detection methods using immobilized mismatch-binding protein have been improved. The present flow-cytometric method for mutation scanning has several important features. It is a single step, no wash assay, capable of rapid and sensitive analysis and, as such, will allow amplified regions to be rapidly scanned for the presence of polymorphisms to prioritize samples for complete sequencing or to detect mutations in known regions of the genome.

Reference will now be made to the present preferred embodiments of the invention, examples of which are shown in the accompanying drawings. Turning now to the Figures, Fig. 1a is a schematic representation of molecules of MutS, which bind to SNP locations in DNA, immobilized on a microsphere in the presence of DNA strands having SNPs and strands of normal DNA, while FIG. 1b is a schematic representation of the binding to the microsphere of the DNA strands which have SNPs as a result of the mismatch-binding protein immobilized thereon. The normal DNA strands remain in solution. DNA having SNPs can then be detected using flow cytometry.

Mismatch-binding proteins include the bacterial mismatch-binding protein MutS, homologues thereof, the T4 bacteriophage enzyme endonuclease VII, one of its functional homologues, or any other protein that recognizes DNA base pair mismatches and which can be immobilized on microspheres by physical adsorption or by the use of an affinity tag which binds to an affinity partner immobilized on the microspheres. Useful affinity tags and binding partners are listed in the TABLE.

TABLE. Affinity Tags and Binding Partners

Affinity Tag	Binding Partner
Biotin	avidin/streptavidin
Hexahistidine	Metal chelates
Glutathione-S-transferase	Glutathione
Maltose binding protein	Maltose
Cellulose binding domain	Cellulose
Protein A	IgG

The microspheres are composed of polystyrene, cellulose, agarose, or other suitable material, and bear one of the affinity tag binding partners listed in the TABLE.

Fluorescently labeled DNA is prepared by amplification using the polymerase chain reaction with fluorescently labeled primers or fluorescently labeled nucleotide triphosphates, or by post-amplification staining with a fluorescent DNA binding dye. Alternatively, the DNA can be labeled using biotinylated nucleotide triphosphates in a polymerase chain reaction amplification process, followed by binding with fluorescently labeled avidin, after the DNA has been fixed onto microspheres by the mismatch-binding protein coating thereon.

Having generally described the present invention, the following EXAMPLE is intended to provide more specific details thereof.

EXAMPLE

Microspheres bearing immobilized mismatch-binding protein are prepared by adsorbing one nanomole of mismatch-binding protein on one hundred million microspheres, each ten micrometers in diameter. The incubation occurs efficiently in a total volume of one milliliter of Tris buffer (50 mM Tris, 100 mM NaCl, pH 8.0) chilled for 12 hours using ice, followed by washing which includes two cycles of centrifugation and resuspension. The DNA samples to be tested are amplified by polymerase chain reaction (PCR) using a high fidelity thermostable DNA polymerase, such as *Pfu*, to optimize amplification fidelity.

Fluorescein-labeled dUTP is included in the amplification reaction such that the amplified DNA contains the fluorescent label. The amplified DNA is then melted and allowed to reanneal. Microspheres bearing immobilized mismatch-binding protein are added (one hundred thousand to a total volume of twenty microliters), and mismatch-containing double-stranded DNA is allowed to bind to the microspheres. The samples are then diluted with buffer to a final volume of five hundred microliters and the microsphere-associated fluorescence is measured in a conventional flow cytometer. Figure 2 is a graph of the fluorescence, as measured by flow cytometry, of immobilized mismatch-binding protein-coated microspheres as a function of the concentration of mismatch-containing DNA (open circles) or non-mismatch-containing DNA (black circles) after 30 min. of co-incubation. As can be seen from Fig. 2, at DNA concentrations greater than 100 nM, the specific binding of the mismatch-containing DNA is clearly distinguishable from the background of normal DNA.

The foregoing description of the invention has been presented for purposes of illustration and description and is not intended to be exhaustive or to limit the invention to the precise form disclosed, and obviously many modifications and variations are possible in light of the above teaching. The embodiments were chosen and described in order to best explain the principles of the invention and its practical application to thereby enable others skilled in the art to best utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated. It is intended that the scope of the invention be defined by the claims appended hereto.

WHAT IS CLAIMED IS:

1. A method for detecting mismatch-containing DNA, which comprises the steps of:

(a) fluorescently labeling the DNA under investigation;

(b) preparing microspheres bearing immobilized mismatch-binding protein;

(c) incubating the fluorescently labeled sample of DNA with the microspheres having mismatch-binding protein immobilized thereon, thereby binding mismatch-containing, fluorescently labeled DNA to the microspheres; and

(d) detecting the mismatch-containing, fluorescently labeled DNA bound to the microspheres by flow cytometry.

2. The method for detecting mismatch-containing DNA as described in claim 1, wherein said step of fluorescently labeling the DNA under investigation is achieved by polymerase chain reaction amplification using a fluorescently labeled primer.

3. The method for detecting mismatch-containing DNA as described in claim 1, wherein said step of fluorescently labeling the DNA under investigation is achieved by polymerase chain reaction amplification in the presence of fluorescent deoxynucleotide triphosphates.

4. The method for detecting mismatch-containing DNA as described in claim 1, wherein said step of fluorescently labeling the DNA under investigation is achieved by using a DNA-binding fluorophore.

5. The method for detecting mismatch-containing DNA as described in claim 1, wherein the mismatch-binding protein is selected from the group consisting of *Escherichia coli* MutS and T4 endonuclease VII.

6. The method for detecting mismatch-containing DNA as described in claim 1, wherein the microspheres are composed from materials selected from the group consisting of polystyrene and cellulose.

7. The method for detecting mismatch-containing DNA as described in claim 1, wherein the mismatch-binding protein is immobilized on the microspheres by physical adsorption.

8. The method for detecting mismatch-containing DNA as described in claim 1, wherein the mismatch-binding protein is immobilized on the microspheres by covalent attachment.

9. The method for detecting mismatch-containing DNA as described in claim 1, wherein the mismatch-binding protein is immobilized on the microsphere by an affinity tag pair selected from the group consisting of avidin-biotin, hexahistidine-metal chelate, and glutathione-S-transferase-glutathione.

10. A method for detecting mismatch-containing DNA which comprises the steps of:

(a) amplifying the DNA under investigation using the polymerase chain reaction in the presence of biotinylated deoxynucleotide triphosphates, thereby producing biotinylated DNA;

(b) preparing microspheres bearing immobilized mismatch-binding protein;

(c) incubating the biotinylated DNA with microspheres bearing the immobilized mismatch-binding protein, thereby binding mismatch-containing, biotinylated DNA to the microspheres;

(d) incubating the microspheres having mismatch-containing, biotinylated DNA bound thereto with fluorescently labeled avidin, thereby producing mismatch-containing, fluorescently labeled DNA bound to the microspheres; and

(e) detecting the mismatch-containing, fluorescently labeled DNA bound to the microspheres by flow cytometry.

11. The method for detecting mismatch-containing DNA as described in claim 10, wherein the mismatch-binding protein is selected from the group consisting of *Escherichia coli* MutS and T4 endonuclease VII.

12. The method for detecting mismatch-containing DNA as described in claim 10, wherein the microspheres are composed from materials selected from the group consisting of polystyrene and cellulose.

13. The method for detecting mismatch-containing DNA as described in claim 10, wherein the mismatch-binding protein is immobilized on the microspheres by physical adsorption.

14. The method for detecting mismatch-containing DNA as described in claim 10, wherein the mismatch-binding protein is immobilized on the microspheres by covalent attachment.

15. The method for detecting mismatch-containing DNA as described in claim 10, wherein the mismatch-binding protein is immobilized on the microsphere by an affinity tag pair selected from the group consisting of avidin-biotin, hexahistidine-metal chelate, and glutathione-S-transferase-glutathione.

16. A method for detecting mismatch-containing DNA, which comprises the steps of:

(a) fluorescently labeling the DNA under investigation;

(b) incubating soluble mismatch-binding protein with the fluorescently labeled DNA, forming thereby a mismatch-binding protein-fluorescently labeled DNA complex;

(b) preparing microspheres adapted to immobilize mismatch-binding protein;

(c) incubating the complex with the microspheres adapted to immobilize mismatch-binding protein, thereby binding mismatch-containing, fluorescently labeled DNA to the microspheres; and

(d) detecting the mismatch-containing, fluorescently labeled DNA bound to the microspheres by flow cytometry.

17. The method for detecting mismatch-containing DNA as described in claim 16, wherein the mismatch-binding protein is selected from the group consisting of *Escherichia coli* MutS and T4 endonuclease VII.

18. The method for detecting mismatch-containing DNA as described in claim 16, wherein the microspheres are composed from materials selected from the group consisting of polystyrene and cellulose.

19. The method for detecting mismatch-containing DNA as described in claim 16, wherein the mismatch-binding protein is immobilized on the microspheres by physical adsorption.

20. The method for detecting mismatch-containing DNA as described in claim 16, wherein the mismatch-binding protein is immobilized on the microspheres by covalent attachment.

21. The method for detecting mismatch-containing DNA as described in claim 16, wherein the mismatch-binding protein is immobilized on the microsphere by an affinity tag pair selected from the group consisting of avidin-biotin, hexahistidine-metal chelate, and glutathione-S-transferase-glutathione.

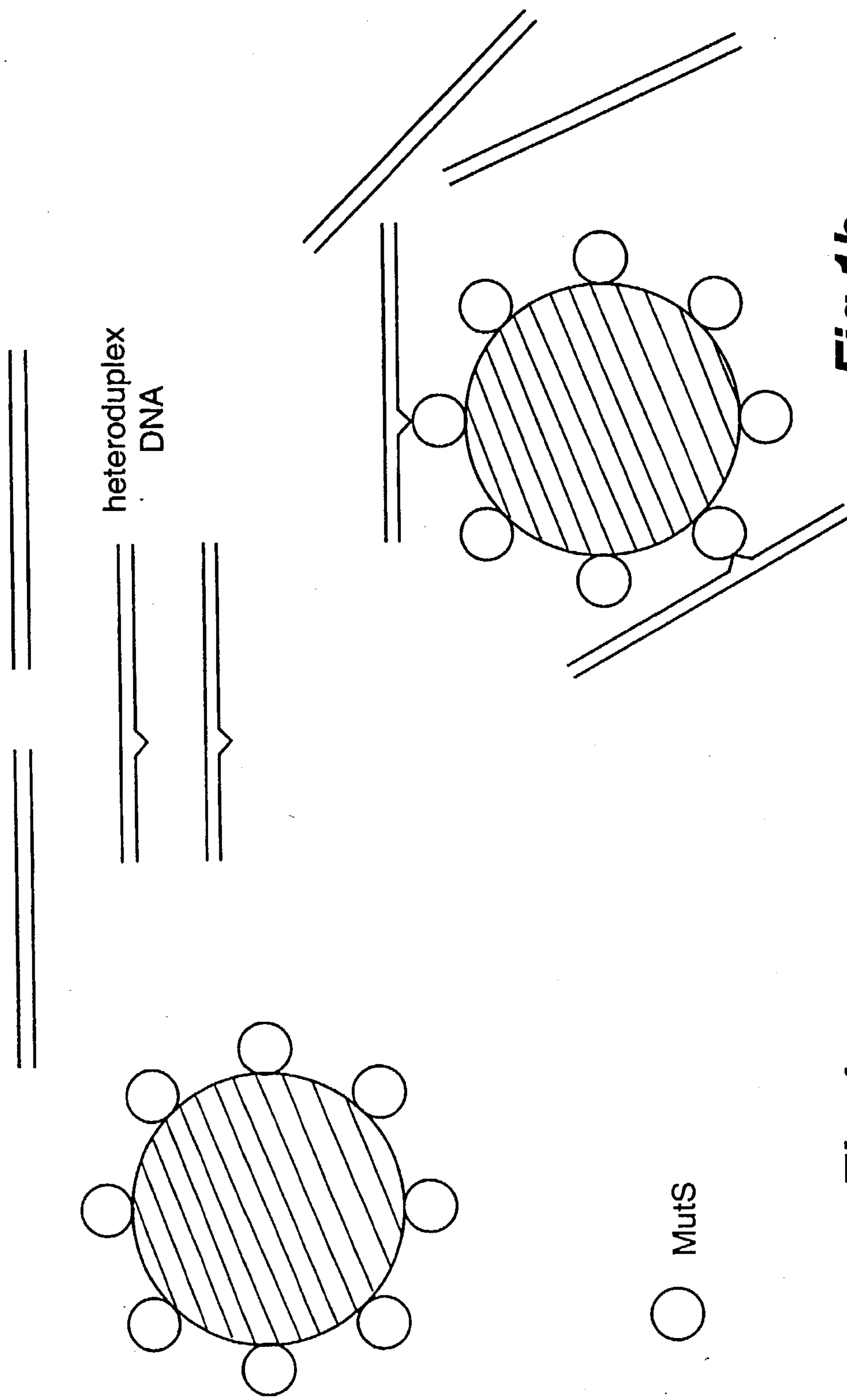

Fig.1b

Fig.1a

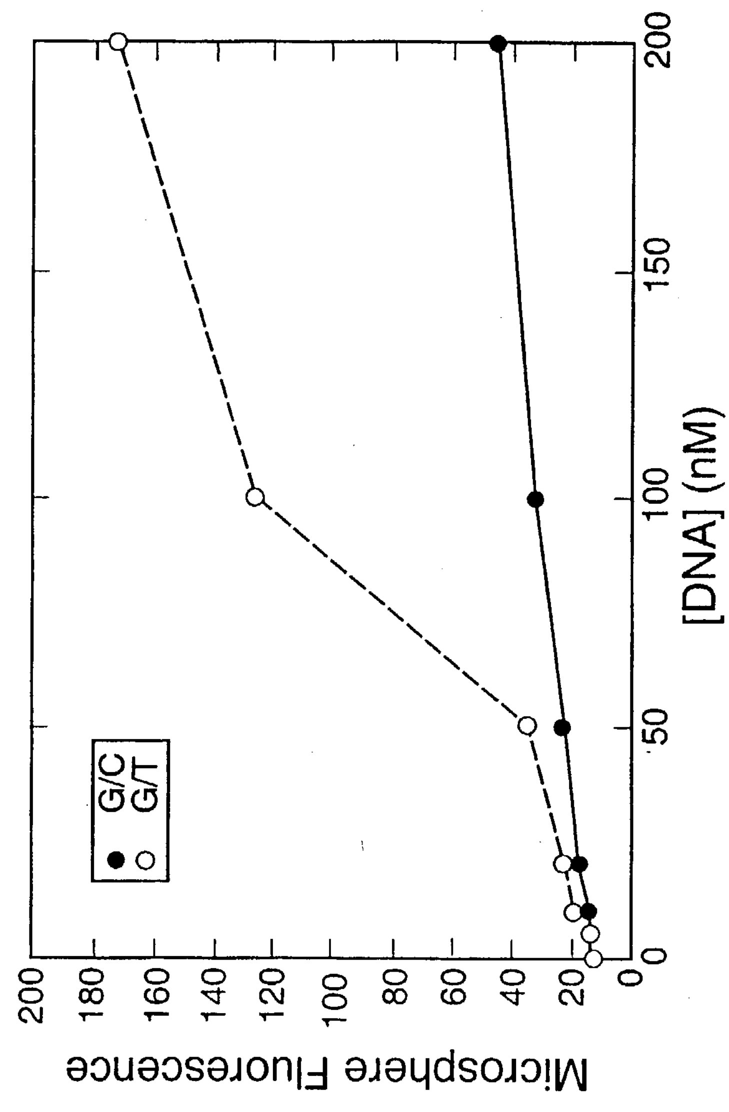
Microsphere Fluorescence
200
180
160
140
120
100
80
60
40
20
0
0
50
100
150
200
[DNA] (nM)
G/C
G/T

Fig. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/23142

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C07H 21/02, 21/04; C12N 15/00
US CL :435/6; 536/23.1, 24.3; 935/76, 77, 78
According to International Patent Classification (IPC) or to both national classification and IPC

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Minimum documentation searched (classification system followed by classification symbols)

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US 5,736,330 A(FULTON et al) 07 April 1998, see the entire document.	1-21
Y	WAGNER et al. Mutation Detection using Immobilized Mismatch Binding Protein (MutS). Nucleic Acids Research. 1995. Vol. 23. No. 19. pages 3944-3948, see the entire document.	1-21
Y	WRIGHT et al. CASTing for Multicomponent DNA-Binding Complexes. Trends in Biochemical Sciences (TIBS). March 1993. Vol. 18. pages 77-80, see the entire document.	1-21
Y	LEW et al. Affinity Selection of Polymerase Chain Reaction Products by DNA-Binding Propteins. Methods in Enzymology. 1993, Vol. 218. pages 526-534, see the entire document.	1-21

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/23142

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THIESEN et al. Target Detection Assay (TDA): a versatile procedure to determine DNA binding sites as demonstrated on SP1 protein. Nucleic Acids Research. 1990. Vol. 18. No. 11. pages 3203-3209.	1-21
Y	US 5,437,976 A (UTERMOHLEN et al.) 01 August 1995, see the entire docunment.	1-21
A	US 5,683,877 (LU-CHANG et al) 04 November 1997, see the entire document.	1-21
A	US 5,683,877 A (LU-CHANG et al.) 04 November 1997, see the entire document.	1-21

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Electronic data bases consulted (Name of data base and where practicable terms used):

USPATFULL, EUROPATFULL, BIOSIS, CAPLUS, MEDLINE

search terms: flow cytometry and (mismatch or mutation) and DNA binding protein